PRODUCTION OF BACITRACIN AND BIOREMEDIATION OF HEAVY METAL FROM *BACILLUS* SPECIES ISOLATED FROM SEWAGE WATER

SYNOPSIS
SUBMITTED

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Supervisor

Dr. VivekBajpai

Submitted by

Yamini Singh Sisodia

Cosupervisor

Dr. Pankaj Kumar Jain

DEPARTMENT OF BIOLOGICAL SCIENCE
Faculty of Arts, Science & Commerce
MODY INSTITUTE OF TECHNOLOGY & SCIENCE
(Deemed University u/s 3 of UGC Act, 1956)
Lakshmangarh-332311, District-Sikar, Rajasthan (India)

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INTRODUCTION:

The term antibiotic appeared as early as 1928 in the French microbiological literature as antibiosis. The phenomenon of antagonism between living organisms was frequently observed even since 1877, when Pasteur and Joubert noticed that aerobic bacteria antagonized the growth of *Bacillus anthracis*. An antibacterial is an agent that inhibits bacterial growth or kills bacteria. The term is often used synonymously with the term *antibiotic(s)*; today, however, with increased knowledge of the causative agents of various infectious diseases, *antibiotic(s)* has come to denote a broader range of antimicrobial compounds, including anti-fungal and other compounds.

The words 'sewage' and 'sewer' come from Old French seuwiere or from Anglo-Norman sewere or from Anglo-French essiver meaning "(channel) to drain the overflow from a fish pond" or "to drain" and ultimately from Vulgar Latin *exaquaticum and *exaquarium, from the verb *exaquare = "to drain", from Latin ex- 'out of' + 'aqua' water (Teresi, 2002). The composition of sludge generated from different geographical locations and individual treatment facilities varies appreciably. However, on average, a typical ton of dry sludge comprises about 80 pounds of nitrogen, 200 pounds of phosphate (P_2O_5), and 10 pounds of potash (K_2O) (Dumontet *et al.*, 2001). These substantial quantities of N and P, in combination with high levels of organic constituents supplying beneficial conditioning properties to soil, highlight the significant value of sewage sludge as a crop fertilizer. Indeed, the application of sludge to arable land enhances crop yield appreciably, commonly exceeding the effects of fertilized controls (Douglas *et al.*, 1991). Sewage sludge typically consists of 41% protein, 25% lipid, 14% carbohydrate and 20% of unknown components on the basis of COD (Tanaka *et al.*, 1997). Sewage sludge commonly contains high amounts of human pathogenic bacteria excreted in faeces and urine. The enteric pathogenic bacterial constituents include *Salmonella* spp., *Listeria* spp., *Escherichia coli* (enterotoxigenic and enter pathogenic variants) *Campylobacter* spp., *Clostridium* spp. and *Yersinia* spp. (Dowdy *et al.*, 1978; Jones, 1980; Larsen *et al.*, 1982; Krovacek, 1989; Skanavis *et al.*, 1994; Strauch, 1998; Jones *et al.*, 2003). A large proportion of these bacteria are both human pathogenic and zoonotic, meaning that they are able to cause infections in both humans and animals. Additionally, these organisms have a strong ability to persistently adapt to changes in the surrounding environment (Kearney *et al.*, 1993) for survival and can be relatively resistant to commonly employed sludge stabilization techniques (Roszak *et al.*, 1987). The health risks related to these

pathogens in terms of spreading sewage sludge on arable land depend on the prior sludge treatments applied, as well as their ability to maintain virulence properties during both storage and distribution on a field used for grazing or food harvesting purposes (Kearney *et al.*, 1993; Jones *et al.*, 2003). These features are usually affected by surrounding environmental factors, including temperature, pH, moisture and nutrient supply (Gerba, 2005).

The term *antibiotic* was first used in 1942 by Selman Waksman and his collaborators in journal articles to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution. This definition excluded substances that kill bacteria, but are not produced by microorganisms (such as gastric juices and hydrogen peroxide). It also excluded synthetic antibacterial compounds such as the sulfonamides. Many antibacterial compounds are relatively small molecules with a molecular weight of less than 2000 atomic mass units. In 1940 Waksman had forecasted, "We are finally approaching a new field of domestication of microorganisms for combating the microbial enemies of man of his domesticated plants and animals. Surely microbiology is entering a new phase of development. During the 1960s, the phase of discovery of antibacterials slackened, but efforts were then made to research also for antifungal, antimycoplasmal. antispirochetal, antiprotozoal, antitumor, antiviral and antiphage compounds, as well as for antibiotics for non-medical uses such as antioxidants. Antibiotics are produced by many microorganisms in various ecological conditions. Producers of antibiotic can be found in rivers, lakes, decaying plants and animal remains etc. but majority of microorganisms that produce antibiotic inhabits soil.

With advances in medicinal chemistry, most of today's antibacterials chemically are semisynthetic modifications of various natural compounds. These include, for example, the beta-lactamantibacterials, which include the penicillins (produced by fungi in the genus *Penicillium*), the cephalosporins, and the carbapenems. Compounds that are still isolated from living organisms are the aminoglycosides, whereas other antibacterials—for example, the sulfonamides, the quinolones, and the oxazolidinones—are produced solely by chemical synthesis. In accordance with this, many antibacterial compounds are classified on the basis of chemical/biosynthetic origin into natural, semisynthetic, and synthetic. Another classification system is based on biological activity; in this classification, antibacterials are divided into two broad groups according to their biological effect on microorganisms: bactericidal agents kill bacteria, and bacteriostatic agents slow down or stall bacterial growth.

Bacitracin was first discovered in 1943 and named after a culture of *Bacillus* and the last name of a seven-year-old American girl, Margaret Tracey; *Bacillus* was isolated from her wounds (Johnson *et al.,* 1945). Many analogues of Bacitracin have been reported by using mass spectroscopy (Morris, 1994). Hundreds of peptide antibiotics have been described in the past half-century (Hancock *et al.,* 1995; Kleinkauf *et al.,* 1988; Perlmanand *et al.,* 1971). These fall into two classes, non-ribosomally synthesized peptides, such as the gramicidins, polymyxins, bacitracins, glycopeptides, etc. and ribosomally synthesized peptides. Non-ribosomal peptides represent a large family of bioactive secondary metabolites produced by bacteria and fungi. Many of these peptides are pharmacologically important drugs like the immunosuppressive cyclosporine A of the antibiotics penicillin, vancomycin and bacitracin. A vast set of substrates like amino acids and hydroxyl as well as carboxylic compounds are known to be incorporated into the peptide chain. In addition, the assembled residues can be further modified by epimerization, N-methylation, acylation, glycosylation or heterocyclization. The final products display linear, cyclic, and/or branched peptide backbones (Doekel *et al.,* 2001; Marahiel *et al.,* 1997). Bacitracin inhibits the formation of bacterial cell wall peptidoglycan by complexing directly with the lipid isoprenyl pyrophosphate (IPP) carrier, inhibiting the dephosphorylation reaction that is required for its regeneration. A typical growth curve for *Bacillus* under Bacitracin production conditions shows fast-growth phase followed by slow-growth phase (Bibel *et al.*, 1978; Laura *et al.*, 2002). For a competitive fermentation advantage, factors like lower capital outlay cost, standard variables and environmental pressure to reduce effluents and cost or variability of substrate and extraction of bacitracin from broth culture should be considered.

OBJECTIVES FOR THE PRESENT STUDY:

1. Isolation and characterization of *Bacillus* strains from sewage treatment plant.
2. Analysis of the susceptibility pattern of different isolates at various environmental stress conditions and at various concentration of different heavy metals (eg. Fe, Cu, Ni, Mg, Mn, Cd, Hg, Zn).
3. Production of antibiotic Bacitracin from isolated *Bacillus sp.* and estimation of its effect on other pathogenic bacterial cultures.
4. Effect of ultra violet radiations and of different heavy metals on bacitracin production.
5. Comparative assessment of bioremediation efficacy of *Bacillus* species on heavy metals.

REVIEW LITERATURE:

Sewage is water-carried wastes, in either solution or suspension that is intended flow away from a community. Also known as waste water flows; sewage is the used water supply of the community and is characterized by its volume or rate of flow, its physical condition, its chemical constituents and the bacteriological organisms that it contains. Depending on their origin, wastewater can be classed as sanitary, commercial, industrial, agricultural or surface runoff. Wastewater, by its nature, is teaming with microbes. Many of these microbes are necessary for the degradation and stabilization of organic matter and thus are beneficial. On the other hand, wastewater may also contain pathogenic or potentially pathogenic microorganisms, which pose a threat to public health. By definition, a pathogen is an organism capable of inflicting damage on its host. Waterborne and water-related diseases caused by pathogenic microbes are among the most serious threats to public health today. Up to 35% of the potential productivity of developing nations is lost because of waterborne disease. Waterborne diseases whose pathogens are spread by the fecal-oral route (with water as the intermediate medium) can be caused by bacteria. viruses, and parasites (including protozoa, worms, and rotifers).The spent water from residences and institutions, carrying body wastes, washing water, food preparation wastes, laundry wastes and other waste products of normal living are classed as domestic or sanitary sewage. Liquid-carried wastes from stores and service establishments serving the immediate community, termed commercial wastes are included in the sanitary or domestic sewage category if their characteristics are similar to household flows. Wastes that result from an industrial process or the production or manufacture of goods are classed as industrial wastes. Their flows and strengths are usually more varied, intense and concentrated than those of sanitary sewage. Surface runoff, also known as storm flow or overland flow, is that portion of precipitation that runs rapidly over the ground surface to a defined channel. Precipitation absorbs gases and particulates from the atmosphere, dissolves and leaches materials from vegetation and soil, suspends matter from the land, washes spills and debris from urban streets and highways and carries all these pollutants as wastes in its flow to a collection point. Bacteria grow as microcolonies in biofilms (i.e., as clumps of cells) they are protected from bacteriophage, phagocytic eukaryotic microorganisms, antibiotics, toxic heavy metals, and xenobiotics. The diffusion

gradients through these microcolonies protect the inner cells from pollutants in the water, and the outer cells of the microcolony are sacrificed for the good of the population.

Wastewater from all of these sources may carry pathogenic organisms that can transmit diseases to humans and other animals contain organic matter that can cause odour and nuisance problems hold nutrients that may cause eutrophication of receiving water bodies and can lead to ecotoxicology. Proper collection and safe nuisance-free disposal of the liquid wastes of a community are legally recognized as a necessity in an urbanized, industrialized society.

Bacitracin ($C_{66}H_{103}N_{17}O_{16}S$) is a metal dependent complex mixture of cyclic polypeptides that is produced by *Bacillus licheniformis* and *Bacillus subtilis*. Most potent activity is reported for bacitracins A1 and B1 that are major components in the mixture of cyclic polypeptide (Morris, 1994). It is a narrow spectrum antibiotic directed primarily against gram-positive bacteria such as *Staphylococcus* and *Streptococcus* via inhibition of cell wall synthesis but also has a little activity against gram-negative organisms (Brewer and Florey, 1980; Prescott and Baggot, 1993). It is most commonly used in complex with zinc which seems to stabilize the antibiotic complex (Quinlan and Gutteridge, 1989). It is poorly absorbed from the gastrointestinal tract (Donoso *et al.*, 1970) as well as from skin and mucosal surfaces. Absorbed bacitracin is excreted by glomerular filtration (Prescott and Baggot, 1993).

The induced mutation followed by strain selection is a routine practice for improving many bacterial and fungal metabolites that primarily includes antibiotics and enzymes. For this purpose, different radiations (UV, X-rays, and γ-rays) or chemicals such as ethyl N methyl N-nitro N-nitroso guanidine (MNNG), Nitrous acid (HNO_2), ethyl methyl sulphonate (EMS) have been used. However, strain improvement is a trial and error process involving laborious procedures. Ultraviolet and gamma irradiation of bacteria have been used to obtain the *Bacillus licheniformis* mutants giving higher yields (Liyong and Tong, 1988). MNNG is an effective mutagen for inducing permanent genetic changes in microorganisms including bacteria, actinomycetes, yeasts and filamentous fungi (Joan *et al.*, 1980; Adamantin *et al.*, 1997; Nadeem *et al.*, 2001; Haq *et al.*, 2005 and Haq *et al.*, 2008).

METHODOLOGY:

1. Collection of sewage samples

Sewage samples will be collected in sterile 100ml polyethylene plastic bottles with screw cap from Sewage treatment plants located in Rajasthan region and stored at 4^0C for future use.

2. Isolation of Bacterial Strains

1gm of sewage sample will mixed with 10ml of sterile distilled water. An aliquot of 0.1 ml of dilutions for each sewage sample will spread onto nutrient agar plates from the appropriate dilution tubes and incubated at 37^0C. Then we will count bacterial colonies after every 24 hrs. The isolated bacterial colonies will then be identified on the basis of Morphological, Biochemical and Molecular characteristics following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

3. Isolation of Genomic DNA

Total DNA from bacterial samples will be extracted by using CTAB (Cetyl Trimethyl Ammonium Bromide) method or other methods if required. The bacterial suspension culture is suspended in TE buffer than lysozyme is added, after that 10% SDS, Proteinase K, 5M NaCl and CTAB (heated to 65^0C) is added. The lysates will be extracted twice with equal volume of chloroform: isoamyl alcohol (24:1). The lysates will again extracted with equal volume of phenol: chloroform: isoamylalcohol (25:24:1) DNA will be precipitated with 0.6 volume of isopropanol (30 min at -20°C, followed by centrifugation at 12000 rpm for 15 min). The DNA pellet will be washed with cold 70% ethanol twice, vacuum dried, and resuspended in 100 µl of TE buffer (Manen *et al.*, 2005).

PCR (Polymerase chain reaction) will be used to amplify two fragments from the extracted DNA of all samples using specific primer. PCR reactions will be carriedout in 25 µl volumes with general PCR program.

4. Sequencing

Sequencing of PCR product is carried out by using 16S rDNA primer and the sequenced product is submitted to NCBI-BankIt or Sequin.

5. BLAST Analysis

Pair wise sequence analysis will be carried out using the GCG software package (Devereux *et al.*, 1984) or other alignment tools. Database search will be carried out with BLAST*n* or with other BLAST tools (http://www.ncbi. nlm.nih.gov/BLAST). Multiple sequence alignments will be prepared with CLUSTAL-W (http://www.ebi.ac.uk/clustalw). Sequences will be aligned with the CLUSTAL-X program (Thompson *et al.*, 1997) or Clustal-W (www.ebi.ac.uk/clustalw) and phylogenetic trees will be constructed by using the Neighbour-Joining algorithm and viewed with the TreeView program (Page, 1996) or with MEGA version 2.0.

6. Strain improvement by UV irradiation

Bacillus licheniformis was maintained on nutrient agar slants. Cell suspension was prepared from 24-hour culture, centrifuged and washed with phosphate citrate buffer (pH 7.5) twice. It is resuspended in phosphate citrate buffer and diluted to10^{-4}-10^{-6} times, transferred to a sterile petridish and exposed to UV lamp at 250 nm for different time intervals ranging between 5-45 minutes. The distance between lamp and suspension was adjusted at 6cm for each trial. After UV exposure, the cell suspension was transferred on nutrient agar plates. The plates were then incubated overnight at 37°C. Bacitracin production was investigated in comparison with control (cells without exposure). Mutant strains with high bacitracin yield developed from culture of *Bacillus licheniformis* by UV irradiation were separated and sub cultured in slants.

7. Antibiotic Sensitivity Test

The disc diffusion method will be used to determine antibiotic sensitivity of the isolates. Kanamycin, Streptomycin, Ampicillin, Tetracycline and other antibiotics will be used and placed at equidistance. Zone of inhibition will be measured and will be classified as resistance or sensitive isolates (Baurer *et al.*, 1966).

8. Optimal growth conditions with reference to temperature and pH will be determined

Salt resistance will be checked by using NaCl by using different concentration ranging from 1M/ml H_2O to 5M/ml H_2O. Effect of Various carbon sources like sucrose, fructose, dextrose will be checked on the growth pattern of the *bacillus*. The isolates will be grown in Nutrient Broth medium with different pH values (3, 5, 6, 7, 8, 9, and 11) and incubation will be carried out at temp 37°C. The optical density of the growing cultures will be measured by Spectrophotometer at 650 nm.

9. Production of Bacitracin

Twenty five millilitre culture medium contained in 250 mL conical flasks will be autoclaved at 121°C for 20 minutes. The medium then will be inoculated with 5% (v/v), 20 hours old vegetative inoculum of *Bacillus licheniformis* for the production of antibiotic bacitracin. The shake flask cultures will be incubated at 37°C for about 48 hours on rotatory shaker at 200 rpm. The cells will be removed by centrifugation at 10,000 rpm for 10 minutes. The clear supernatant will be collected and then will be used for antibiotic assay to determine bacitracin potency.

10. Bioassay of Bacitracin

The potency of antibiotic bacitracin (IU/mL) will be determined by agar diffusion method William (1977). LB agar medium will be autoclaved and 20 mL of the medium will be poured aseptically in the sterile petri plates and will be allowed to congeal. Inoculum of *Micrococcus luteus* will be spread upon the plate .Well will be dig by the help of a borer and then the wells will then be filled by the isolated bacitracin. Plates were then incubated at 37°C. zone of inhibition will be measured.

SIGNIFICANCE OF THE PRESENT STUDY:

One important observation in this study has to be taken that complete removal of the heavy metal toxicity should remove only within 15 and 21 days also the results will indicate

that the use of native or indigenous micro flora of bioremediation is useful and beneficial than commercial inoculum. These results indicated that, biodegradation of complex hydrocarbon requires only single species of Bacillus. It shows its resistance power against different environmental stress conditions if we may be able to increase its resistance power as well as its capability to detoxify metal toxicity we can make a new approach.

Conventional processes used for removal of heavy metals from industrial and sewage wastewaters include chemical precipitation, oxy reduction, filtration, electrochemical techniques and sophisticated separation processes using membranes. These processes are usually expensive when heavy metals are present in moderate concentrations. This characteristic stimulates the use of alternative biotechnologies, due to their reduced cost and lower aggressiveness to the environment. This work will present some results on the use of microbes from the genus *Bacillus* for the uptake of cadmium, zinc, copper and lead ions.

FUTURE PROSPECTS:

Many heavy metals are known to be toxic and carcinogenic for humans, and their contamination of soils and aquifers is a great environmental concern. Millions tons of hazardous and non-hazardous wastes are generated each year in India. Environmental bioremediation, the science of control of pollution by the use of biological means, has no parallel in terms of elimination of waste, selectivity, and efficiency, especially where the pollutant is a xenobiotic. In general, it can be used either for sequestration or detoxification or degradation of the pollutant. An indigenous bacterial strain, *Bacillus licheniformis*, was discovered to be degrading the pesticide cypermethrin in addition to being capable of utilizing a number of mono aromatics and pesticides. The present study is innovative because of its importance. Rajasthan specially Shekhawati region is facing the problem of water scarcity if we may be able to detoxify waste water and can use this water source for irrigation purpose it will be beneficial for society as well as it will remove soil and water pollution also. The treatment and disposal costs of these wastes are causing a vast amount of financial burden to various industries and may soon outstrip the available resources. Thus, there is a great need to better manage these wastes via reduces, reuse and recycle. We are trying to make a common man capable to degrade heavy metal toxins in water and other contaminated sites by using *bacillus* culture which will be available in pouches in a market if the results will be positive. We are also trying to screen out most resistant strain of *bacillus* against environmental stress conditions so that at any temperature, pH , environment one can use

bioremediation processes are adversely affected and even inhibited by co-contamination of organic pollutants with heavy metals.

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Dr. Vivek Bajpai

9/8/13

Dr. Pankaj Kumar Jain

As per recommendation by Supervisor